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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S): S. Panzner, et al. EXAMINER: G.S. Kishore

SERIAL NO.: 10/081,617 ART UNIT: 1615

FILED: February 21, 2002 CONFIRMATION NO.: 9573

FOR: *AMPHOTERIC LIPOSOMES AND THEIR USE*

CERTIFICATE OF MAILING

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as first class mail in an envelope addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on:

By: Michelle A. Aiello Date: October 18, 2006

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF STEFFEN PANZNER UNDER 37 C.F.R. § 1.132 TRAVERSING GROUNDS OF REJECTION

Under 37 C.F.R. § 1.132 and regarding the rejection of claims 1-3, 5-11, and 21-59, over Hafez et al. (*Biophysical Journal*, 79: 1438-1446, 2000; "Hafez") in view of Huang (U.S. Patent 5,283,122; "Huang") or Lishko et al. (U.S. Patent 5,753,263; "Lishko"), or over Deshmukh et al. (U.S. Patent 6,258,792; "Deshmukh"), I declare:

1. I am an inventor of the subject matter that is described and claimed in the above-captioned patent application and fully familiar with the facts therein.

2. I have been working in the field of liposomes for more than 10 years and have been studying liposomal drug delivery for over 6 years.

3. I have written numerous patents and patent applications in the field and I am frequently presenting work in the field of liposomal drug delivery at industry and scientific conferences, e.g. the annual "Liposome Days" of the International Liposome Society, "Nucleic

Acid World Summit" of Strategic Research Institute, "Eurotides", a European meeting for oligonucleotides and the therapeutic delivery of such substances and other related occasions.

4. The amphoteric liposomes of this invention are distinct from both the anionic and cationic liposomes described variously by U.S. Patent 5,283,122 to Huang ("Huang"), U.S. Patent 5,753,263 to Lishko *et al.*; ("Lishko"), and U.S. Patent 6,258,792 to Deshmukh ("Deshmukh"). The cationic and anionic liposomes of the cited prior art are pH-sensitive meaning that the charge of the entire liposome varies. However, these systems are restricted to either a gain-of-charge (i.e., neutral to charged) or a loss-of-charge (charged to neutral) and are capable of adopting only one charged state—positive <u>or</u> negative. Charge on the amphoteric liposomes of this invention, by contrast, can be positive, negative, or neutral, depending upon pH.

5. The amphoteric liposomes of the invention are also different from the lipid mixtures described in Hafez *et al.*, *Biophysical Journal*, 79: 1438-1446, 2000 ("Hafez") in that the Hafez liposomes lack neutral lipids.

6. The teachings of Hafez show destabilization of a lamellar lipid phase after a change of the pH of the medium. The systems disclosed in Hafez comprise lipid mixtures being stable at neutral pH (DODAC/CHEMS in various ratios) that undergo continuous fusion when exposed to acidic pH conditions (Hafez, Figure 2).

7. In another system of Hafez, comprising DC-Chol and DOPA with ratios >1.6, a stable lamellar phase can be produced at acidic pH which undergoes continuous fusion when exposed to higher pH (Hafez, Figure 4).

8. In yet another system of Hafez, liposomes from DC-Chol and DOPA were produced with ratios between 1.1 and 1.6 at basic pH which undergo continuous fusion when exposed to lower pH (Hafez, p.1441, right column).

9. In summary, Hafez teaches mixtures of anionic and cationic lipids that undergo continuous fusion once the surface charge of such mixtures is reduced to zero.

10. In contrast, we have surprisingly discovered that liposomes of the present invention, comprising neutral lipids as well as anionic and cationic lipids, are stable both at a first low pH and a second high pH and can transition from the first pH to the second pH without significant fusion.

11. We also have surprisingly discovered that the transfection efficiency (a functional measure) of amphoteric liposomes is significantly enhanced upon the addition of neutral lipids.

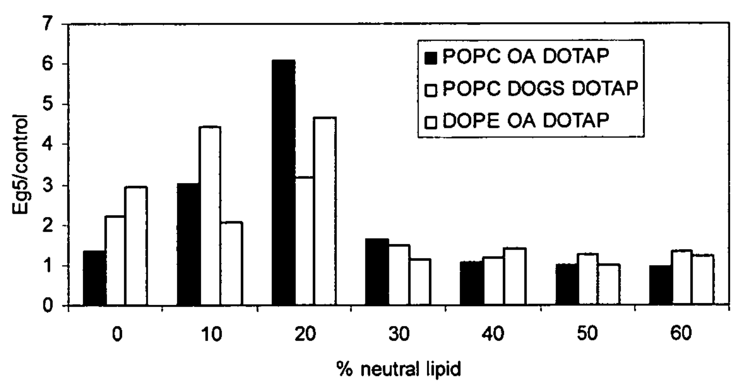
12. We measured the transfection efficiency of various amphoteric liposomal formulations loaded with an antisense oligonucleotide targeting Eg5; a protein critical for mitosis. Eg5 inhibition results in the arrest of cell division and induces apoptosis.

13. Fifty microliters (50 µl) of the various lipid mixtures in ethanol were placed into individual wells of a 96 well multi-titer plate. Next, 115 µl of a buffer (HAc 10mM, NaCl 50mM, pH 4.5) containing an Eg5 antisense oligonucleotide was added and rapidly mixed with the lipids resulting in a final ratio between the cationic lipid and backbone phosphate charges of the oligonucleotide of between 2 and about 5. The resulting solution was about 30% ethanolic and in a subsequent step the pH was adjusted using 750 µl Na_2HPO_4 27mM, NaCl 140mM, pH 8.0. The final dispersion was about 5.5% ethanol at a pH of 7.5.

14. For the following experiments, HeLa cells were grown to about 25% confluence in DMEM with 10% fetal calf serum (FCS) at 37°C and 5% CO_2. For transfection, the medium was changed to Optimem and the various liposomal formulations were added to a final oligonucleotide concentration of 15nM. Positive controls were performed using Oligofectamin according to manufacturer instructions to transfect the Eg5 antisense oligonucleotide at a concentration of 30nM oligonucleotide. An unrelated oligonucleotide that does not interfere with cell division was also used and had no effect on cell survival. After a 4 hour incubation, the medium was completed by adding FCS in DMEM and cells were grown for 3 days. Living cells were quantified using Cell Titer Blue. The survival of untransfected cells was set at 100% and the survival of cells transfected with Eg5 using Oligofectamin was set at 0%. Growth inhibition for cells transfected with the various liposomal formulations is expressed as %inhibition (Eg5) / %inhibition (control oligo).

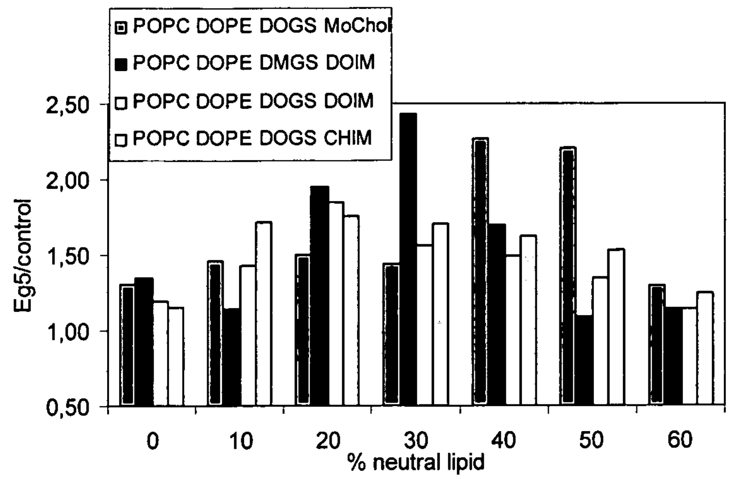
15. *Experiment #1*: Amphoteric liposomes were formulated using a strong cation (DOTAP), a weak anion (oleic acid (OA) or dioleoylglycerol hemisuccinate (DOGS)), and increasing amounts of a neutral lipid (POPC or DOPE). The ratio of the anion:cation was maintained at 1.5-2.0 to 1 in all formulations. For all three formulations, transfection was significantly enhanced with the addition of 10-20% neutral lipid. This experiment demonstrates that the chemical nature of the membrane anchor for the lipids does not influence the fusogenicity of the liposomes in the presence of neutral lipids. OA is a monocarboxylic acid grafted onto a monoalkyl chain, whereas DOGS has the same charged moeity, but is combined with a diacylglycerol anchor.

16. The experiment shows that addition of either a phosphatidylcholine or a phosphatidylethanolamine are beneficial for the transfection efficacy of the amphoteric liposomes.

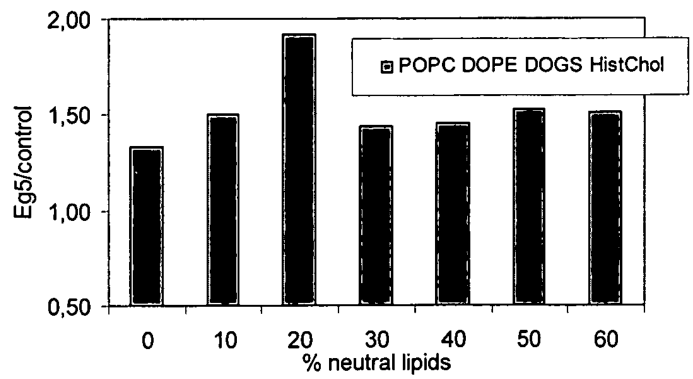


17. *Experiment #2*: Amphoteric liposomes were formulated using a weak cation (MoChol, DOIM, or CHIM), a weak anion (DOGS, or dimyristoylglycerol hemisuccinate (DMGS)), and a combination of neutral lipids (POPC and DOPE). The ratio of the anion:cation was maintained at about 2:3 in all formulations. Here, transfection was significantly enhanced with the addition of 20-50% neutral lipids, depending upon the anion/cation combination. Higher concentrations of neutral lipid do not enhance liposomal transfection. This experiment demonstrates that the chemical nature of the membrane anchor for the lipids does not influence

the fusogenicity of the liposomes in the presence of neutral lipids. MoChol and CHIM are based on cholesterol whereas the other lipids are based on diacylglycerol. Also, saturated lipid components (DMGS) or unsaturated (DOIM and DOGS) components react similarly to the inclusion of neutral lipids.



18. *Experiment #3*: Amphoteric liposomes were formulated using the weak anionic lipid DOGS and the amphoteric lipid HistChol at a ratio of 2:1 with increasing concentrations of a neutral lipid mixture of POPC and DOPE. Maximum transfection efficiency was achieved at 20% neutral lipids. This experiment further demonstrates that mixtures comprising genuine amphoteric lipids likewise benefit from the addition of neutral lipid in terms of functional cell transfection.



19. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Oct. 19th, 2006

Date

Steffen Panzner, Ph.D.

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